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ORIGINAL ARTICLE

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Postlabeling detection of DNA adducts of antitumor alkylating agents

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Abstract The sensitivity for DNA adduct formation by antitumor alkylating agents (mechlorethamine, cisplatin and adozelesin) of the postlabeling technique and thin-layer chromatography was studied. Three DNAs were used: a double-stranded 20-bp oligonucleotide of defined sequence, calf thymus DNA and murine leukemia L1210 cellular DNA. With high concentrations of mechlorethamine, there was a marked decrease in normal dGp, a lesser decrease in dAp and dCp and no change in dTp. Using 2D mapping PEI-cellulose thin-layer chromatography analyses, it was found that six mechlorethamine: DNA adducts were produced after a short exposure to mechlorethamine. After an extended time at relatively high drug concentrations there was an alteration in the mechlorethamine: DNA adduct pattern that may reflect the conversion of monoadducts to crosslinked adducts. Similar observations were made with cisplatin and adozelesin. When murine leukemia L1210 cells were treated with 50 μ*M* mechlorethamine or 50 μ*M* cisplatin for 1 h, six or more mechlorethamine: DNA adducts and five cisplatin: DNA adducts were detected. After allowing 6 h. for repair of potentially lethal damage, several adducts were no longer detectable and others appeared with diminished intensity. Nuclease P_1 dephosphorylates normal nucleotides at relatively low enzyme concentrations with variation depending upon the nucleotide. In general, considerably lower concentrations of nuclease P_1 were required to dephosphorylate the normal nucleotides than to dephosphorylate the antitumor alkylating agent: nucleotide adducts, thus allowing increased sensitivity of the postlabeling assay. The sensitivity of detection of antitumor alkylating agent: DNA adducts in DNA from treated L1210 cells approached one adduct per 10^7–10^8 nucleotides. These results suggest that the postlabeling technique may be sufficiently sensitive and specific for the study of the clinically effective levels of antitumor alkylating agents.

Key words Postlabeling · Alkylating agents · DNA adducts

Introduction

The antitumor alkylating agents, including platinum-based anticancer drugs, are among the most effective chemotherapeutic agents in clinical use [7]. Current clinical investigations involving the antitumor alkylating agents emphasize: (1) the development of more effective and selective analogs, especially new platinum-containing agents; (2) dose intensification regimens, because the antitumor alkylating agents have steep dose/tumor cell killing patterns and because the myelosuppression produced by these agents is now clinically manageable [13]; and (3) combinations with modulators to improve the effectiveness of the antitumor alkylating agents, the modulators including hypoxic cell sensitizers, topoisomerase II inhibitors, oxygen delivery agents and antiangiogenic agents [2, 44]. The multiple variables inherent in clinical trials relating to understanding the effects in patients of new antitumor alkylating agents, high dose and the addition of adjuvants to standard agents are major and prohibitive if only classical phase II and III study endpoints such as frequency, magnitude and duration of response, and survival are available. An appropriate 'surrogate' endpoint reflecting alkylating agent efficacy would be highly desirable and valuable to the clinical investigator.

The most widely accepted mechanism of antitumor alkylating agent cytotoxicity is damage to genomic

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DNA by formation of drug adducts, particularly bifunctional adducts on the genomic DNA molecule (inter- and/or intrastrand crosslinking of DNA) [7]. The qualitative and quantitative determination of alkylating agent: DNA adducts could provide a very useful surrogate endpoint of clinical effect.

The postlabeling technique was developed to detect chemical carcinogen-induced bulky DNA adducts [2, 37, 39]. Briefly, the technique involves the digestion of DNA into normal and carcinogen-bound dNps (dXps) by micrococcal endonuclease and spleen phophodiesterase enzymes. The enzymatically digested DNA (dNps + dXps) is then treated with nuclease P_1 which selectively dephosphorylates the dNps. The carcinogen bound dNps (dXps) are then radioactively labeled with [γ-^{32}P]-ATP using T_4 kinase. The radiolabeled dXps are chromatographically separated, identified and quantitated. This technique can detect one carcinogen–DNA adduct in 10^7–10^{10} nucleotides. It has been employed successfully for the study of DNA damage by chemical carcinogens [2, 37, 39], free radicals and radiation [38], anticancer drugs [23, 33], indigenous DNA modification [27] and smoking-induced adducts in the placenta [10]. Other techniques for the study of DNA damage include alkaline elution [20, 25], immunochemistry [31, 45, 46], transfected human shuttle vector containing alkylated sequences [15], PCR analysis of stop sites corresponding to crosslinking [5, 6, 43] and transcriptional analysis of alkylation [14].

In addition to a clinical surrogate endpoint, DNA postlabeling applied to antitumor alkylating agents could be a useful laboratory tool for the study of the cellular pharmacology of alkylating agents, including DNA repair, and the frequency, distribution and nature of drug: DNA adducts. Altered DNA repair is presumed to be one of the most common and important mechanisms of tumor resistance to the antitumor alkylating agents. The current study was designed to evaluate the effectiveness of the postlabeling technique for determining the drug: DNA adducts produced by three antitumor agents that represent different classes of antitumor alkylating agents: mechlorethamine (HN_2) [4, 19, 24, 30, 35, 47], cisplatin [8, 9, 11, 26, 28] and adozelesin [3, 22, 49, 50].

Materials and methods

Reagents

Two 20-bp oligonucleotides of specific sequence (strand A TACAGTAATCTGATATAAAT; strand B ATGTCATTAGACTATATTTA) were synthesized in the Dana-Farber Cancer Institute core facility. These oligonucleotide sequences were developed to provide several sites for efficient inter- and intrastrand crosslinking by the study drugs. Calf thymus DNA, *cis*-diamminedichloroplatinum(II) (cisplatin), HN_2, 2′-deoxyadenosine 3′-monophosphate (dAp), 2′-deoxycytidine 3′-monophosphate (dCp), 2′-deoxythymidine 3′-monophosphate (dTp), 2′-deoxyguanosine 3′-monophosphate (dGp) were purchased from Sigma Chemical Co. (St. Louis, Mo). Adozelesin was generously provided as a gift by the Upjohn Co. (Kalamazoo, Mich.). [γ-^{32}P]-ATP (10 mCi/ml) was purchased from DuPont New England Nuclear (Boston, Mass). Polyethyleneimine–cellulose (PEl-cellulose) thin-layer chromatography sheets were prepared in our laboratory according to the method of Randerath et al. [19, 37, 38] or purchased from Brinkmann Instruments (Westbury, N.Y.). DuPont Cronex X-ray films and Lightning Plus intensifying screens were purchased from DuPont (Atlanta, Ga).

Enzymes

Micrococcal endonuclease (MN) from *Staphylococcus aureus*, nuclease P_1 from *Penicillium citrinum*, potato apyrase, RNase A and RNase T_1 were purchased from Sigma. Spleen phosphodiesterase (SPD) and proteinase K were purchased from Boehringer Mannheim (Indianapolis, Ind.). T_4 polynucleotide kinase (PNK) and PNK buffer were purchased from U.S. Biochemical Corporation (Cleveland, Ohio).

In vitro studies

Equal amounts of the two 20 bp oligonucleotide strands (0.2 μg/μl) were annealed for 5 min at 65°C and then slowly cooled to room temperature. The double-stranded DNA oligonucleotide (2 μg) was reacted with concentrations of HN_2 ranging from 0.4 μ*M* to 400 m*M* in 50 m*M* sodium phosphate buffer (pH 7.4) for various times (0, 1, 2, 4, 8, 12, 24 or 48 h.) at 37°C. The nucleic acid was isolated from the reaction mixture by precipitation with ice-cold 5.0 *M* NaCl (1 vol) and absolute ethanol (4 vols). The alkylation products of the oligonucleotide drug reaction were determined by 5′-end labeling using [γ-^{32}P]-ATP and T_4 PNK followed by electrophoresis on 12% polyacrylamide gel under denaturing conditions.

Calf thymus DNA (10 μg) was reacted with 4 m*M* HN_2, 40 μ*M* cisplatin or 50 μ*M* adozelesin in phosphate buffer (pH 7.4) for 10 h at 37°C. The nucleic acid was isolated by precipitation using ice-cold 5.0 *M* NaCl (1 vol) and absolute ethanol (4 vols), followed by centrifugation. The DNA pellet was washed by resuspension in ice-cold 80% ethanol and stored at − 20°C.

L1210 cell studies

Murine leukemia L1210 cells were grown in suspension in RPMI medium containing 10% horse serum and antibiotics. For experiments 10^7 cells were exposed to 50 μ*M* HN_2 or 50 μ*M* cisplatin for 1 h at 37°C in serum-free medium. After drug exposure the cells were quick-frozen or were returned to depleted medium containing serum and maintained at 37°C to allow potentially lethal damage repair for 6 h. [1, 21, 48], then quick frozen.

Cellular DNA was isolated using a conventional phenol/chloroform extraction method [42]. Briefly, L1210 cells (10^7) suspended in 2 ml 5% sodium dodecyl sulfate solution containing 5 m*M* EDTA, 1 *M* Tris-HCl (pH 8.0), 360 μg RNase A and 100 U RNase T_1 were incubated for 45 min at 37°C. Protein was removed by extraction of the cell lysate with saturated phenol and chloroformamylalcohol (24:1 v/v). The cellular DNA was then isolated by precipitation using 5 *M* NaCl and ice-cold absolute ethanol as described above. The quality and quantity of the cellular DNA was determined by the ultraviolet absorption ratio (260/280 nm). The purified DNA was stored at − 20°C.

Postlabeling technique

The postlabeling technique was similar to that described previously [2, 16, 37, 39].

The 20-bp oligonucleotide HN_2 and cisplatin reaction products (2 μg) were enzymatically digested by addition to a solution containing final concentrations of 30 m*M* sodium succinate, 10 m*M* $CaCl_2$, 0.01 U/μl MN and 0.5 μg/μl SPD (pH 6.0) for 2.5 h at 37°C. For the enzymatic digestion, the calf thymus DNA (10 μg) HN_2, cisplatin or adozelesin reaction products and L1210 cellular DNA (10 μg) products were suspended in a solution containing 0.5 U/μl MN and 0.5 μg/μl SPD and incubated at 37°C for 4 h.

The enzymatic digestion products were diluted to 1 dNp ng/μl (1.94 p*M* dNp/μl). A 6-μl aliquot of each sample was reacted with 0.5 μl [γ-^{32}P]-ATP (10 mCi/ml) in 2.0 μl PNK buffer (200 m*M* Bicine, 100 m*M* DL-dithiothreitol, 10 m*M* spermidine, 100 m*M* $MgCl_2$, pH 9.6) (0.1 μl/μl sample), 0.5 U/μl T4 PNK in a volume of 10 μl at 37°C for 40 min, then further incubated with 2 μl apyrase (40 mU/μl) for 30 min at 37°C to remove unreacted [γ-^{32}P]-ATP. Radioactively labeled samples were stored at − 80°C for subsequent analysis.

Nuclease P_1 treatment

Nuclease P_1 efficiently converts normal 2′-deoxyribonucleoside 3′-monophosphates to deoxyribonucleosides but reacts less efficiently at sites of bulky adducts such as chemical carcinogen-bound nucleotides [37, 38]. A concentration of nuclease P_1 was selected that would dephosphorylate approximately 50–90% of normal dNps since antitumor alkylating agent adducts especially monoadducts have been shown to be labile in high concentrations of nuclease P_1 (> 0.1 μg/μl) (unpublished observations). Thus, a nuclease P_1 concentration of 7.5 ng/μl was found to be optimal for treatment of the 20 bp oligonucleotide product mixture and a nuclease P_1 concentration of 50 ng/μl was optimal for dephosphorylation of the calf thymus and L1210 cellular DNA reaction products (10 μg DNA). The nuclease P_1 reaction contained of 1 m*M* $ZnCl_2$, 1 *M* sodium acetate, pH 5.0, and the reaction was carried on for 40 min at 37°C. The reaction was stopped by the addition of 3 μl 500 m*M* 2-*N*-cyclohexylaminoethanesulfonic acid (CHES), pH 9.8. [γ-^{32}P]-ATP labeling was performed as described above.

Thin-layer chromatography and autoradiography

PEI-cellulose thin-layer chromatography sheets were washed with distilled water and air-dried [16]. ^{32}P-labeled samples (*pdNp + *pdXp) were diluted with 20 m*M* CHES (pH 9.8) at a dilution of 1:30. The diluted samples (5 μl) were applied to the PEI-cellulose thin-layer chromatography sheets 2.0 cm from the lower edge and 2.0 cm from lefthand edge. Elution was carried out in either 1.5 *M* ammonium formate, pH 3.4, or 0.28 *M* $(NH_4)_2SO_4$, 50 m*M* NaH_2PO_4, pH 6.8. The analyses of two-dimensional(2D)mapping were performed by eluting samples for 14 cm in 1.5 *M* ammonium formate, pH 3.4, rinsing the thin-layer sheet in water for 5 min and allowing the sheet to air-dry, then rotating the chromatogram by 90° and eluting in 0.28 *M* $(NH_4)_2SO_4$, 50 m*M* NaH_2PO_4, pH 6.8, for 15 cm, followed by air-drying. The thin-layer chromatography sheets were visualized by exposure to DuPont Cronex X-ray film with an intensifying screen for 1–3 h at room temperature. For quantitation, radiolabeled spots on the 2D-mapping PEI-cellulose thin-layer chromatography sheets were located by autoradiography, cut out and the Cherenkov radiation (Beckman LS 500) determined. The relative adduct labeling (RAL) was calculated [39] as:

$$\mathrm{RAL} = \frac{\text{Alkylated adducts (cpm)}}{\text{Total nucleotides (cpm)}}$$

If P_1 nuclease was used, the specific activity of [γ-^{32}P]-ATP was determined by labeling 1 pmol dAp and the calculation adjusted as:

$$\mathrm{RAL}' = \frac{\text{Alkylated adducts (cpm)}}{\text{*pdAp(cpm/pmol)} \times \text{DNA(μg)} \times 3240\ \text{pmol/μg}}$$

based on the assumptions that the tested adduct was absolutely resistant to P_1 nuclease and that 1 μg DNA is equal to 3240 pmol dNp.

Results

The structures of the antitumor alkylating agents and the sequences of the 20-bp oligonucleotides are shown in Fig. 1.

Mechlorethamine adducts in vitro

The double stranded 20-bp oligonucleotide was exposed to 1 *M* HN_2 in phosphate buffer (pH 7.4) for 1 h at 37°C. Postlabeling was performed on the reaction product mixture. The samples were analyzed using 2D-mapping PEI-cellulose thin layer chromatography as described in Materials and methods. The reaction with HN_2 produced six adduct spots in addition to the four normal dNps (Fig. 2). Exposure to this high concentration of HN_2 reduced the dGp, dCp and dAp cpm values to 8%, 10% and 72%, respectively, of the control values. The RAL of total alkylation products was 29% over total nucleotides, consistent with other published results [4, 12, 17, 19, 24, 30, 47].

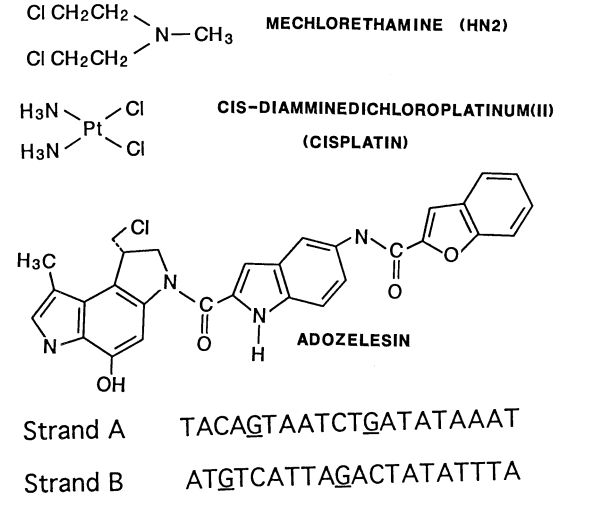

Fig. 1 Structures of the three antitumor alkylating agents and sequences of 20-bp oligonucleotides

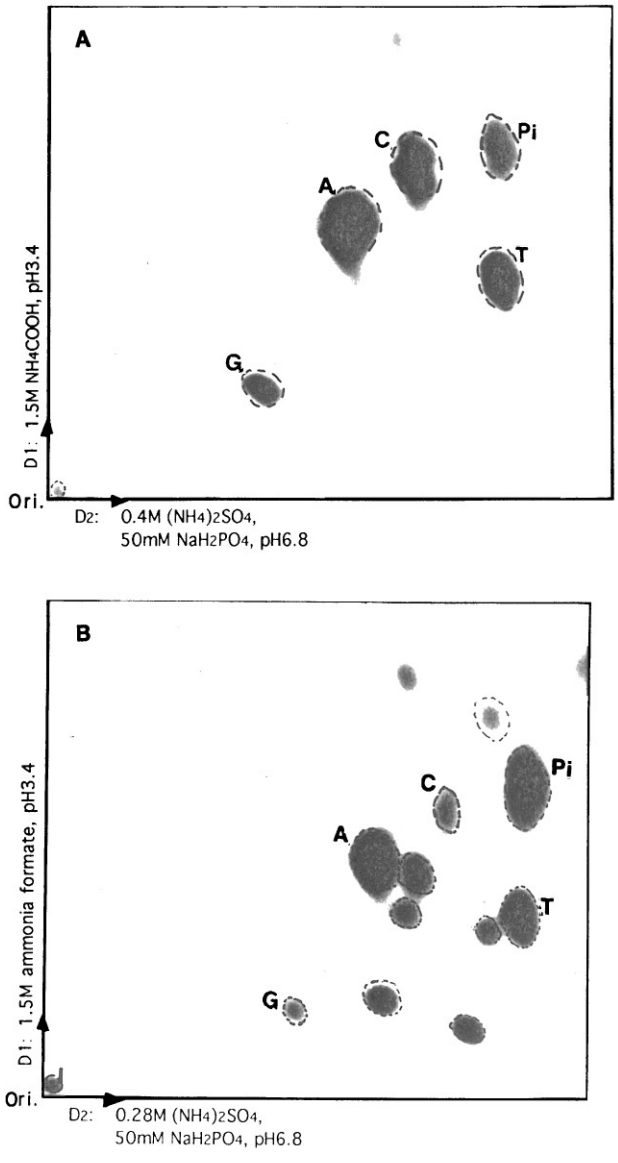

Fig. 2A, B 2D chromatograms of equal amounts of the double-stranded 20-bp oligonucleotide without treatment (**A**) or after exposure to 1 *M* HN_2 in phosphate buffer at pH 7.4 for 1 h. at 37°C (**B**). Samples were analyzed by 2D-mapping PEI-cellulose thin layer chromatography. The individual normal dNps and adduct spots were determined by Cherenkov radiation (*T*, *C*, *A*, *G* normal *pdNps. *Pi* orthophosphate). The six labeled spots in **B** represent HN_2: DNA adducts

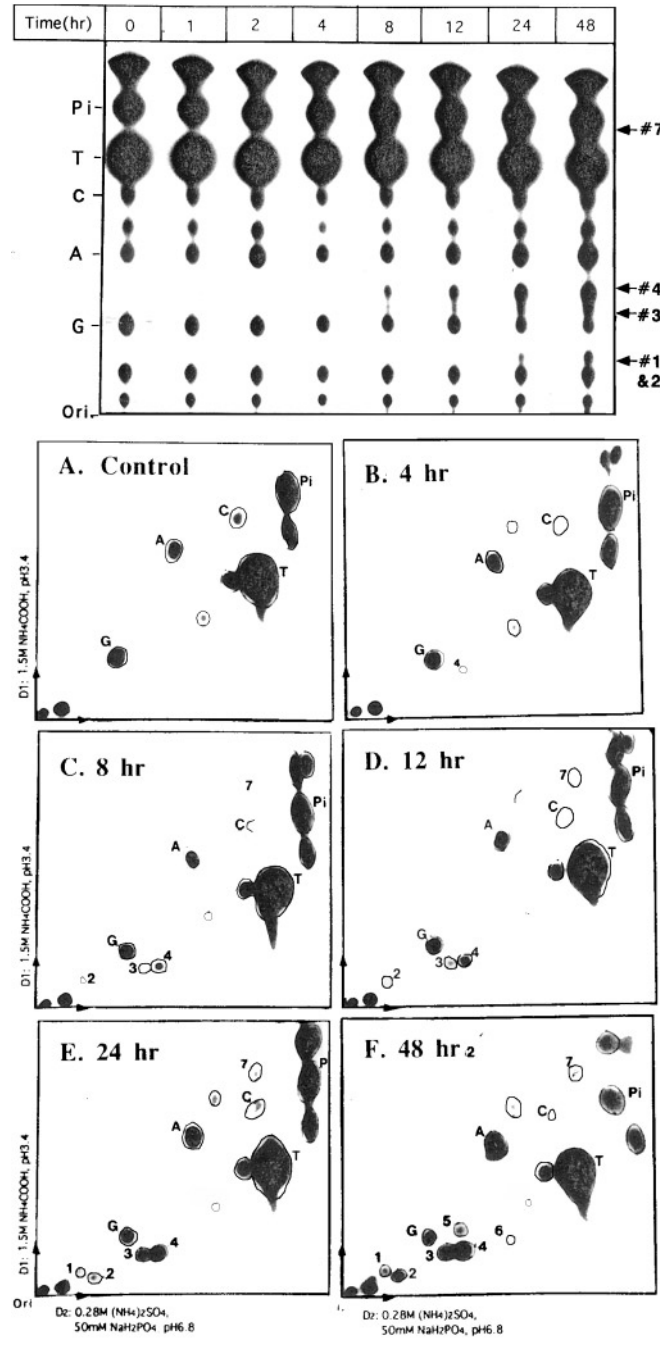

Fig. 3 1D and 2D chromatograms of the double-stranded 20-bp oligonucleotide exposed to 4 m*M* HN_2 for 1, 2, 4, 8, 12, 24 or 48 h. The postlabeling reaction and analysis were performed in the presence of nuclease P_1 (7.5 ng/μl). *Upper panel*: *T*, *C*, *A* and *G* on the left side indicate the positions of four normal dNps. Arrows on the right side represent HN_2: DNA adducts. Unlabeled spots are due to the impurity of the labeling materials. *Lower panel*: *G*, *A*, *C* and *T* are the four normal dNps. The numbers 1 to 7 represent HN_2: DNA adducts

Effect of duration of exposure to mechlorethamine on DNA adducts in vitro

The double-stranded 20-bp oligonucleotide was exposed to 4 m*M* HN_2 for various time periods (Fig. 3). ^{32}P-labeled samples from each reaction were eluted on PEI-cellulose thin-layer chromatography sheets and developed by 1D and 2D mapping. Up to seven HN_2: DNA adducts were detectable by 48 h. The products were numbered 1 to 7 in accordance with their positions from the bottom to the top on the 2D-mapping chromatogram. Products #5 and #6 were of low intensity and appeared only after 48 h. of incubation with HN_2. The quantitative analyses of these chromatograms is shown in Fig. 4. For this analysis

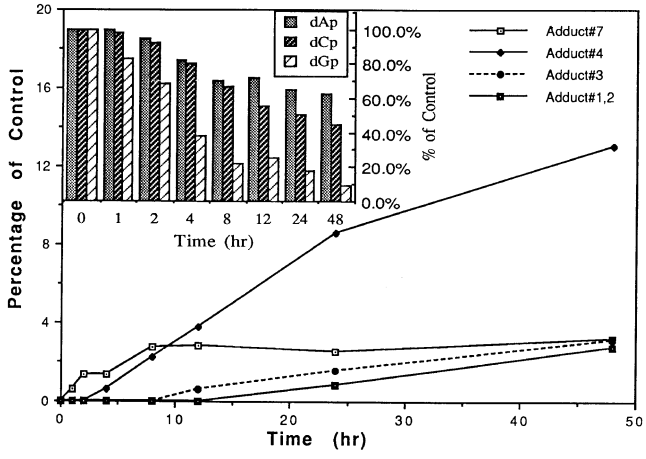

Fig. 4 Kinetics of HN_2: DNA adduct formation and normal dNp disappearance for the reaction of the double-stranded 20-bp oligonucleotide with 4 m*M* HN_2. The appearance of HN_2: DNA adducts is presented as the percentage of control (*pdTp at 0 h). For the *insert*, the dNps from nontreated double-stranded 20-bp oligonucleotide were used as controls set at 100%

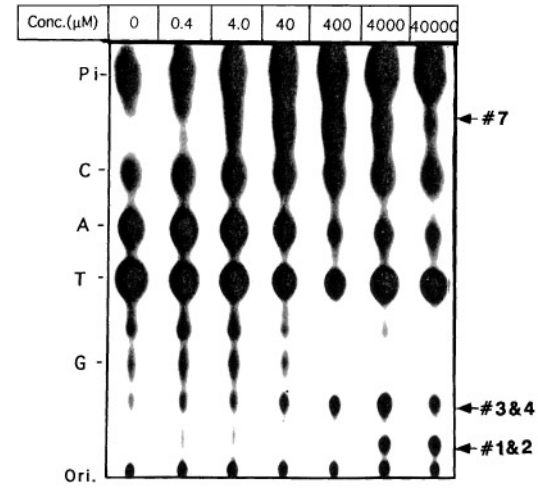

Fig. 5 1D thin-layer chromatogram of the double-stranded 20-bp oligonucleotide after exposure to various concentrations of HN_2 for 10 h. Postlabeling was performed in the presence of nuclease P_1 (7.5 ng/μl). *T*, *C*, *A* and *G* indicate the positions of four normal dNps. The labeled *arrows* represent the HN_2: DNA adducts described in Figs. 3 and 4

*pdTp (cpm) at 0 h was used as a control because dTp was assumed not to react with HN_2. The earliest adduct (spot #7) peaked at 3% of control at 8 h and remained essentially the same thereafter. The other early adduct (spot #4) progressively increased reaching peak values of 8.6% and 13.0% after 24 and 48 h. incubation. Another adduct (spot #3) was visible after 8 h of incubation reaching a peak of 3% after 48 h. The adduct (spot #1 + 2) was visible after 12 h. and increased to 2.7% of control after 48 h of incubation. The adducts (spots #5 and #6) were visible only after 48 h. of incubation.

Effect of concentration of mechlorethamine on DNA adducts

The double-stranded 20-bp oligonucleotide was exposed to concentrations of HN_2 from 0.4 μ*M* to 40 m*M* for 10 h at 37°C. ^{32}P-labeled samples were separated by 1D PEI-cellulose thin-layer chromatography as shown in Fig. 5. The intensity (frequency) of the HN_2: DNA adducts, compared to the four normal dNps, appeared to increase with increasing concentration of HN_2. The HN_2: DNA adduct labeled #7 was most intense at the HN_2 concentrations of 4 μ*M*, 40 μ*M* and 400 μ*M* (Fig. 5), while the adduct HN_2: DNA labeled #3&4 slowly increased with drug concentrations of 400 μ*M* and 4 m*M* then decreased at 40 m*M*. The HN_2: DNA adduct labeled #1&2 was visible only at relatively high HN_2 concentrations. At higher concentrations (4 m*M* or greater) a decrease occurred in the HN_2: DNA adduct labeled #7 while an increase occurred in the intensity in the HN_2: DNA adduct spots #3&4 and #1&2 in a reciprocal fashion, suggesting that the adduct #7 could be HN_2: DNA monoadduct while the HN_2: DNA adducts labeled #3&4 and #1&2 may represent crosslinking of the DNA.

Comparison of DNA adduct formation by three antitumor alkylating agents in vitro

Calf thymus DNA (10 μg) was exposed to 4 m*M* HN_2, 40 μ*M* cisplatin or 50 μ*M* adozelesin in the phosphate buffer (pH 7.4) for 10 h at 37°C followed by postlabeling in the presence of nuclease P_1 and 2D-mapping on PEI-cellulose thin-layer chromatography. For these antitumor alkylating agent exposures in the absence of nuclease P_1 treatment, no drug: DNA adducts were detectable; however, the alkylating agent exposure changed the ratio of four normal dNps of calf thymus DNA (Fig. 6). Exposure to HN_2 reduced dGp, dAp and dCp cpm to 11%, 38% and 45% of untreated controls, as was found with the 20-bp oligonucleotide. Exposure to cisplatin decreased dGp by 41% and dAp by 18% compared to the untreated controls. Adozelesin preferentially reacted with dAp reducing its level to 18% of the untreated controls. However, adozelesin treatment also decreased dCp to approximately 20% of the control.

In the presence of nuclease P_1 at 50 ng/μl, the calf thymus control DNA demonstrated normal dNps in the positions similar to those found for the 20-bp oligonucleotide and calf thymus DNA in the absence of nuclease P_1 (compare Figs. 2A, 3B and 6A). At this low concentration of nuclease P_1, some differential 3'-dephosphorylation of dNps took place. Thus, 3'-dephosphorylation of dCp approached 99%, dAp 85% and

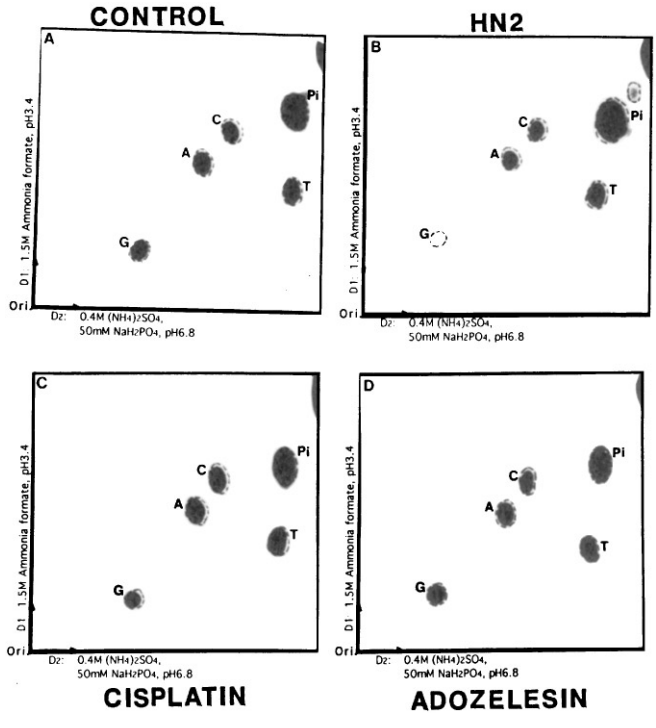

Fig. 6A–D 2D thin-layer chromatogram of calf thymus DNA untreated or after exposure to 4 m*M* HN_2, 40 μ*M* cisplatin or 50 μ*M* adozelesin for 10 h. Postlabeling was performed in the absence of P_1 nuclease. **A** Autoradiogram of control calf thymus DNA; **B** autoradiogram of DNA exposed to 4 m*M* HN_2; **C** autoradiogram of DNA exposed to 40 μ*M* cisplatin; **D** autoradiogram of DNA exposed to 50 μ*M* adozelesin

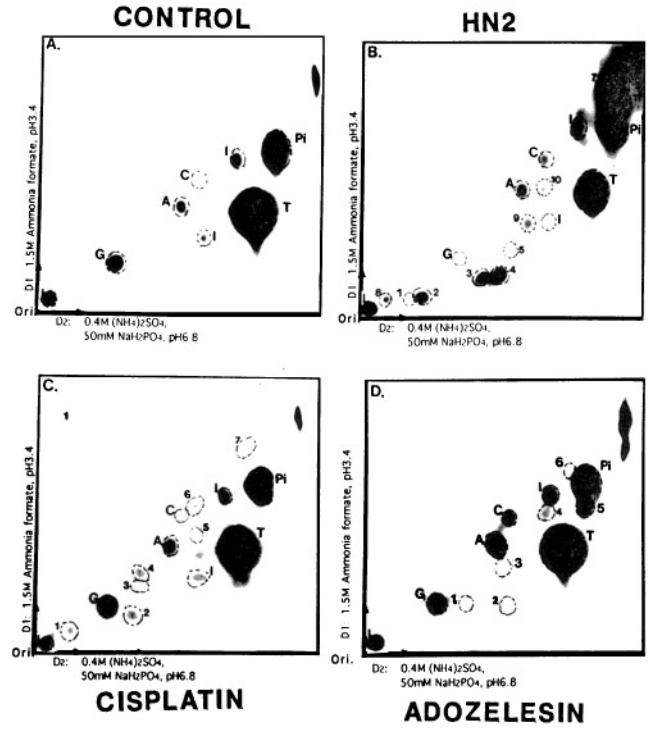

Fig. 7A–D 2D thin-layer chromatogram of calf thymus DNA untreated or after exposure to 4 m*M* HN_2, 40 μ*M* cisplatin or 50 μ*M* adozelesin for 10 h. Postlabeling was performed in the presence of nuclease P_1 (50 ng/μl). **A** Autoradiogram of untreated control calf thymus DNA (*I* represents an impurity from radiolabeling materials or calf thymus DNA); **B** autoradiogram of DNA treated with 4 m*M* HN_2. **C** autoradiogram of DNA treated with 40 μ*M* cisplatin; **D** autoradiogram of DNA treated with 50 μ*M* adozelesin

dGp 60% (compare Figs. 6A and 7A). Three additional spots labeled 'I' (impurity) possibly from labeling materials or some trace RNA in the commercial calf thymus DNA were also visible [9].

A 2D chromatogram showing HN_2: DNA adducts produced in calf thymus DNA treated with HN_2 (4 m*M*, 10 h) is presented in Fig. 7B. All HN_2: DNA adducts seen with the 20-bp oligonucleotide were observed except adduct (spot #6) (compare Figs 3B and 7B). Four additional adducts (spots #8–#11) were visible. The intensity (frequency) of each adduct was calculated according to the method of Randerath [4] (Table 1). The RAL′ was approximately 1–10 HN_2: DNA alkylated adducts per 10^6 nucleotides. Reaction of calf thymus DNA with cisplatin (40 μ*M*) produced seven drug: DNA adducts that were clearly distinct from those seen with HN_2 (Fig. 7C). The RAL′ of the cisplatin: DNA adducts were relatively low, perhaps because a lower concentration of cisplatin (40 μ*M*) was used than HN_2 (4 m*M*) (Table 1). Reaction of calf thymus DNA with adozelesin produced six adducts (Fig. 7D). Adozelesin did not react with single dAp or with the single stranded 20-bp oligonucleotide, but did react with double stranded 20-bp oligonucleotide (data not shown), suggesting that this drug requires

Table 1 The number and relative intensity of drug:DNA adducts produced in vitro by reaction of each of three antitumor alkylating agents with calf thymus DNA. Calf thymus DNA (10 μg) was treated with mechlorethamine 4 m*M*, cisplatin 40 μ*M* and adozelesin 40 μg/μl for 10 h at 37°C. Postlabeling and thin-layer chromatography were performed in the presence of nuclease P_1 (50 ng/μl). Relative adduct labeling (*RAL*) was calculated as follows:

$$\mathrm{RAL}' = \frac{\mathrm{d^*p} \times \mathrm{p\ (cpm)}}{\mathrm{d^*pAp\ (cpm)} \times 3.24 \times 10^5}$$

The specific activity of [^{32}P]-ATP was determined by labeling 1 pmol of dAp. The data are presented as the number of adducts per 10^6 nucleotides (*ND* = not determined)

Mechlorethamine		Cisplatin		Adozelesin	
Adduct	RAL′	Adduct	RAL′	Adduct	RAL′
M1	1.01	C1	1.79	A1	1.13
M2	2.95	C2	2.26	A2	1.91
M3	2.89	C3	1.64	A3	1.75
M4	4.38	C4	2.00	A4	2.30
M5	1.68	C5	1.45	A5	3.59
M6	ND	C6	2.51	A6	1.93
M7	10.38	C7	2.60		
M8	1.24				
M9	2.00				
M10	1.80				
M11	6.88				

Table 2 Comparison of the DNA binding characteristics observed, decreases in normal nucleotides and intensity (frequency) of drug: DNA adduct formation by three antitumor alkylating agents reacted with calf thymus DNA in vitro. Postlabeling was performed in the presence of P_1 nuclease (50 ng/μl) for 10 μg DNA and 1 pmol of 3′-dAp was used for the determination of specific activity of [^{32}P]-ATP (*NC* no change)

	Mechlorethamine	Cisplatin	Adozelesin
Reaction site[a]	G(N7) > A(N7)	G(N7) > A(N7)	A(N3)
Groove[a]	Major	Major	Minor
Crosslinking[a]	Inter > intra	Intra > inter	Monoligand
Adduct spots[b]	7–11	4–7	3–6
dGp (%)[c]	11.5%	58.6%	NC
dAp (%)	62.1%	82.2%	82.2%
dCp (%)	55.3%	NC	80.5%
RAL′[d]	35.2	14.3	12.6

[a]DNA reaction site, DNA groove binding preference and DNA crosslinking tendencies have been described previously [3, 5–7, 9, 50]

[b]The number of adduct spots depends upon the structure of the DNA and the alkylating agent exposure, i.e. concentration, temperature and duration

[c]% dNps is based on postlabeling from calf thymus DNA treated with antitumor alkylating agent expressed as a percentage of control. S.D. = 10% of triplicates of cpm in two experiments

[d]$$\mathrm{RAL}' = \frac{\text{total d*p} \times \text{p (cpm)}}{\text{d*pAp (cpm)} \times 3.24 \times 10^5}$$

appropriate secondary structure for DNA binding [22, 49, 50]. The adozelesin: DNA adducts were more stable at high concentrations of nuclease P_1 than were the HN_2: DNA adducts and cisplatin: DNA adducts. Table 2 summarizes some DNA binding characteristics of the three antitumor alkylating agents.

Antitumor alkylating agent: DNA adducts detected in murine leukemia L1210 cells

The murine leukemia L1210 cell line is a common model in cancer therapeutic research. This cell line is in general, sensitive to antitumor alkylating agents. L1210 cells in suspension culture were treated with 50 μ*M* HN_2 or cisplatin for 1 h. at 37°C and DNA was isolated immediately or following a 6-h period to allow repair of potentially lethal damage from the drugs. A 1D post labeling chromatogram of L1210 cellular DNA after antitumor alkylating agent treatment is shown in Fig. 8. Five HN_2: DNA adducts were visible compared with the control and the RAL was 3–200 adducts per 10^{10} nucleotides. The level of overall adduct formation after the 6-h holding period was two- to five-fold less than immediately after drug treatment, suggesting a substantial degree of repair. There were six cisplatin: DNA adducts immediately after drug treatment, but only two after the 6 h holding period, again indicating a significant capacity of these cells to repair cisplatin

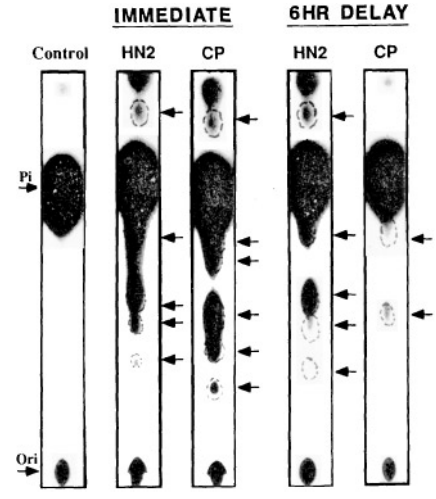

Fig. 8 1D thin-layer chromatogram of murine leukemia L1210 cellular DNA untreated or after exposure of the cells to 50 μ*M* HN2 or 50 μ*M* cisplatin for 1 h. followed by immediate DNA isolation or a 6-h. delay period prior to DNA isolation. Postlabeling was performed using 10 μg DNA in the presence of nuclease P_1 (50 ng/μl). The *arrows* on the right side of the chromatograms represent the alkylated adducts

Table 3 DNA adducts in cultured mouse leukemia L1210 cells produced by cisplatin. Cultured mouse leukemia L1210 cells were treated with 50 μ*M* cisplatin for 1 h. at 37°C. Adducts were determined by postlabeling in the presence of P_1 nuclease (60 ng/μl) and analyzed by 2D PEI-cellulose thin-layer chromatography

Adduct	Adduct cpm ($\times 10^{-3}$)	Adducts/DNA nucleotide[a] ($\times 10^9$)
a	251 ± 24	156 ± 15
b	5 ± 2	3 ± 1
c	7 ± 1	4 ± 1
d	2 ± 0	1 ± 0
e	235 ± 21	146 ± 13
f	254 ± 18	157 ± 11

[a]The counts per minute obtained when 1 pmol dAp was labeled was 3.98×10^9 (cpm/pmol); since 0.125 μg of DNA was analyzed, the adducts/DNA nucleotide values were calculated by dividing the adduct counts per minute by 1.61×10^{12}

damage to DNA. In addition, as shown in the 2D chromatograms in Fig. 9, six cisplatin: DNA adduct spots (a–f) were observed in treated L1210 cells compared with control cells when the cellular DNA was isolated immediately after drug exposure. The abundance of these adducts ranged from 2 to 250 adducts per 10^8 nucleotides (Table 3). There were also two spots in addition to Pi in the control cells (Fig. 9A). One was residual pdTp due to the limited action of nuclease P_1 on this nucleotide at 60 ng/μl (data not shown). The other spot may be an 'endogenous' DNA adduct from leukemia cells, or it may represent impurities from the labeling materials.

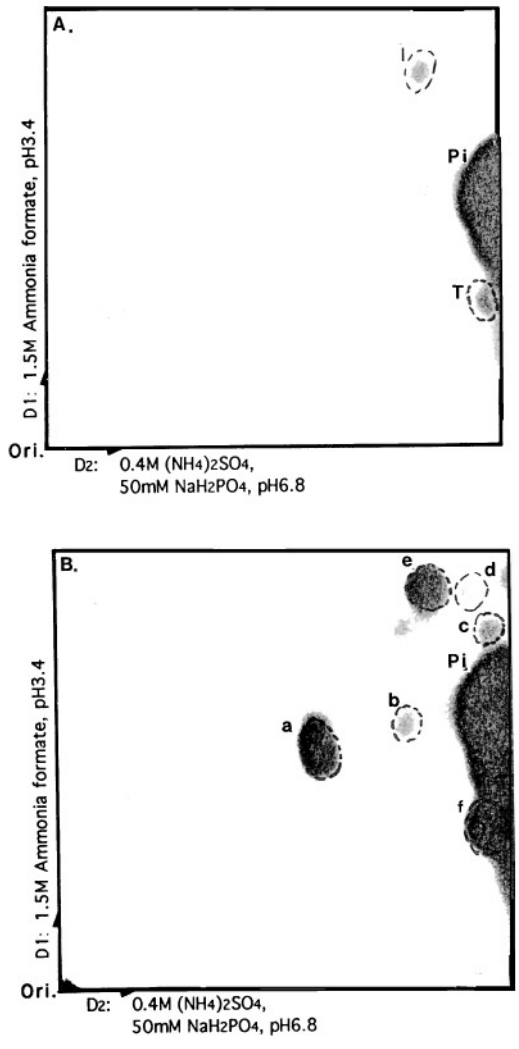

Fig. 9A, B Pt-DNA adducts detected in mouse leukemia L1210 cells. L1210 cells were treated with 50 μ*M* cisplatin for 1 h. at 37°C and DNA was isolated. Postlabeling was then performed in the presence of 60 ng/μl P_1 nuclease and the products were analyzed by 2D mapping. The individual adduct spots were quantitated by Cherenkov counting. **A** Control; **B** cells treated with 50 μ*M* cisplatin (*Pi* orthophosphate)

Discussion

A number of techniques have been employed to study DNA damage and repair, crosslinking, mutagenesis and carcinogenesis [2, 5, 6, 10, 14, 15, 25, 27, 31, 32, 33, 36–41, 43, 45, 46]. After careful review of these techniques it appeared that the postlabeling technique, because of its sensitivity and specificity, might be adaptable for use as a clinical 'surrogate' endpoint. The ultimate objectives of our research are: (1) to employ the number of adducts as a surrogate endpoint for DNA damage in the context of clinical trials and (2) to study the mechanisms of sensitivity and resistance to clinically important antitumor alkylating agents at a DNA level. The purpose of our current study was to adapt the postlabeling technique to the determination of drug: DNA adducts produced by DNA-binding antitumor agents in model systems.

The number of drug: DNA adducts produced by HN_2 was drug concentration- and time-dependent. At low drug concentrations and for short periods of time, it is likely, and our data suggest, that mono-HN_2-dNp adducts were formed in DNA in the in vitro reaction model. After exposure to higher HN_2 concentrations and/or longer exposure times more bifunctional HN_2-dNp adducts, that is intra- or interstrand crosslinking products, are formed. All of these drug: DNA adducts are subject to DNA repair mechanisms in cells [7]. Our observations in L1210 cells are consistent with DNA repair. Based largely on these time and concentration sequences in drug: DNA in vitro reaction models, we postulate that HN_2: DNA adducts corresponding to #1–#4, and #8 could be bifunctional crosslinking and that adducts #7, #9–#11 may be monofunctional adducts (Figs. 3B and 7B). For the reaction of cisplatin with DNA, the drug: DNA adducts #1–#4 may be crosslinking and the adducts #5–#7 monofunctional adducts (Fig. 7C). Adozelesin is a DNA site-directed alkylating agent which covalently binds to dAp [3, 22, 49, 50]. We posit that adozelesin: DNA adducts #1–#3 may be adducts of adozelesin with DNA while adducts #4–#5 may be the byproducts of hydrolyzed adducts [Fig. 7D). It is possible that the drug: DNA adducts corresponding to spots #1 to #4 for HN_2, shown under the conditions of longer exposure and higher concentrations, were from decomposed products of drug: DNA adducts because of high instability of alkylated adducts particularly the N-7 alkylated dGp [34, 41, 51]. The secondary structure of DNA plays an important role in the formation of drug adducts. For example, our postlabeling studies showed that HN_2 and cisplatin reacted with single dNps but adozelesin did not. Adozelesin also did not react with the single stranded 20-bp oligonucleotide but required double stranded DNA for the adduct formation. HN_2 produced greater adduct formation with double-stranded 20-bp oligonucleotide than with the single-stranded 20-bp oligonucleotide, consistent with this drug's well-known capacity for interstrand crosslinking.

Alkylating agents, including cisplatin, are clinically the most effective class of antitumor agents [7]. We have initiated studies with antitumor alkylating agents in mouse leukemia L1210 cells because these cells are sensitive to antitumor alkylating agents, easy to grow both in culture and in animals, and have served as a major preclinical model in cancer therapeutic research. At a concentration of HN_2 or cisplatin sufficient to kill 99–99.9% of the cells, the drug: DNA adducts were readily detected by the postlabeling technique followed by PEI-cellulose thin-layer chromatography. The sensitivity of detection was as little as one

drug adduct per 10^9–10^{10} nucleotides. Drug: DNA adduct detection, especially for cisplatin, was significantly decreased if the cells were maintained in depleted medium for 6 h prior to DNA isolation compared with immediate DNA isolation after the 1-h drug exposure, suggesting substantial DNA repair occurred during the delay period [1, 21, 48]. Consistent with this, the surviving fraction for L1210 cells exposed to cisplatin (50 μM) for 1 h with immediate subculture was 0.0018 and after the 6-h delay period to allow potentially lethal damage repair the surviving fraction increased to 0.006. Similarly, L1210 cells exposed for 1 h to HN_2 (50 μM) with immediate subculture had a surviving fraction of 0.0005. When the cells were maintained in depleted medium for 6 h after HN_2 exposure to allow potentially lethal damage repair to occur, the surviving fraction increased to 0.007 [1, 21, 48]. Our data also suggested that the repair of the DNA intrastrand crosslinking produced by cisplatin might be faster than the repair of the DNA interstrand crosslinking produced by HN_2 (Fig. 8).

Randerath and his colleagues were the first to establish the postlabeling technique followed by PEI-cellulose thin-layer chromatographic analysis to assess DNA damage [37]. These investigators were able to further increase the sensitivity of the postlabeling technique to about one adduct in 10^9–10^{10} nucleotides by the addition of nuclease P_1 treatment to the assay [39]. While nuclease P_1 at concentrations > 0.5 μg/μl was appropriate for digestion of normal dNp in reaction mixtures containing 'bulky' chemical carcinogen: DNA adducts, the current study demonstrated, as suggested by others, that the HN_2: DNA adducts were labile to such high concentrations of nuclease P_1 [18, 29, 33]. In these antitumor alkylating agent experiments the optimal concentrations of nuclease P_1 were 7 ng/μl for assays containing 2 μg double-stranded 20-bp oligonucleotide and 50 ng/μl for assays containing 10 μg calf thymus or cellular DNA. These nuclease P_1 concentrations produced minimal loss of drug: DNA adducts while allowing 50–90% dephosphorylation of the normal dNps. There was selectivity for the normal dNps by nuclease P_1 under these conditions, but 3′-dephosphorylation of dCp and dAp occurred to a greater extent than 3′-dephosphorylation dGp and dTp (Figs. 6A and 7A).

To maximize the sensitivity and specificity of the postlabeling technique for clinically important antitumor alkylating agents our current studies emphasize the following aims: (1) the identification of the major adducts; (2) the definition of more precise utility of the nuclease P_1 treatment in the differential dephosphorylation of selected adducts from normal nucleotides; (3) the development of standards that would make it possible with image-enhancement techniques to increase the sensitivity of the assay; and (4) the determination of the type and number of adducts.

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References

1. Barranco SC, Townsend CM Jr (1986) Loss in cell killing effectiveness of anticancer drugs in human grastric cancer clones due to recovery from potentially lethal damage in vitro. Cancer Res 46:623
2. Beach AC, Gupta RC (1992) Human biomonitoring and the ^{32}P-postlabeling assay. Carcinogenesis 13:1053
3. Bhuyan BK, Smith KS, Adams EG, Petzold GL, McGovren JP (1992) Lethality, DNA alkylation and cell cycle effects of adozelesin (U-73975) on rodent and human cells. Cancer Res 52:5687
4. Brookes P, Lawley PD (1961) The reaction of mono- and difunctional alkylating agents with nucleic acid. Biochem J 80:496
5. Bubley GJ, Ara G, Ashburner BP, Balk SP, Varshney A, Teicher BA (1992) Identification of *cis*-diamminedichloro-platinum(II) adducts in DNA from drug-treated human cell nuclei. Biotechniques 12:694
6. Bubley GJ, Teicher BA, Ogata GK, Sandoval LS, Kusomoto T (1994) Differences in in vivo and in vitro sequence-specific sites of cisplatin-DNA adduct formation and detection of a dose-response relationship. Biochem Pharmacol 48:145
7. Colvin M (1993) Alkylating agents and platinum antitumor compounds. In: Holland JF, Frei EI, Bast RC Jr, Kufe DW, Morton DL, Weichselbaum RR (eds) Cancer medicine. Lea & Febiger, Philadelphia, p 733
8. Eastman A (1983) Characterization of the adducts produced in DNA by cis-diamminedichloroplatinum(II) and cis-dichloro (ethylenediamine) platinum(II). Biochemistry 22:3927
9. Eastman A (1987) The formation, isolation and characterization of DNA adducts produced by anti cancer platinum complexes. Pharmacol Ther 34:155
10. Everson RB, Randerath E, Santella RM, Cefalo RC, Avitts TA, Randerath K (1986) Detection of smoking-related covalent DNA adducts in human placenta. Science 231:54
11. Fichtinger-Schepman AMJ, Van der Veer JL, den Harteg JHJ, Lohman PHM, Reedijk J (1985) Adducts of the antitumor drug cis-diamminedichloroplatinum(II) with DNA: formation, identification, and quantitation. Biochemistry 24:707
12. Försti A, Hemminki K (1994) A ^{32}P-postlabelling assay for DNA adducts induced by *cis*-diamminedichloroplatinum(II). Cancer Lett 83:129
13. Frei E III (1992) Pharmacologic strategies for high-dose chemotherapy. In: Armitage JO, Antman KH (eds) High-dose cancer therapy: pharmacology, hematopoietins, stem cells. Williams & WIlkins, Baltimore, p 3
14. Gray PJ, Cullinane C, Phillips DR (1991) In vitro transcription analysis of DNA alkylation by nitrogen mustard. Biochemistry 30:8036
15. Grueneberg DA, Ojwang JO, Benasuttim M, Hartman S, Loechler EI (1991) Construction of a human shuttle vector containing a single nitrogen mustard interstrand, DNA-DNA cross-link at a unique plasmid location. Cancer Res 51:2268
16. Gupta RC, Reddy MV, Randerath K (1983) ^{32}P-postlabeling anaylsis of non-radioactive aromatic carcinogen-DNA adducts. Carcinogenesis 3:1081
17. Hemminki K (1985) Binding of metabolites of cyclophosphamide to DNA in a rat liver microsomal system and in vivo in mice. Cancer Res 45:4237
18. Hemminki K (1987) DNA-binding products of nor-nitrogen mustard, a metabolite of cyclophosphamide. Chem Biol Interact 61:75
19. Hemminki K, Kallama S (1986) Reactions of nitromustards with DNA. IARC Science Publications 78:55

20. Hengstler JG, Fuchs J, Oesch F (1992) DNA strand breaks and DNA cross-links in peripheral mononuclear blood cells of ovarian cancer patients during chemotherapy with cyclophosphamide/carboplatin. Cancer Res 52:5622
21. Holden SA, Teicher BA, Boeheim K, Weichselbaum RR, Ervin TJ (1987) Platinum complexes inhibit repair of potentially lethal damage following bleomycin treatment. Br J Cancer 55:245
22. Hurley LH, Reynolds VL, Swenson DH, Petzold GL, Scahill TA (1984) Reaction of the antitumor antibiotic CC-1065 with DNA: structure of a DNA adduct with DNA sequence specificity. Science 226:843
23. Kato S, Yamashita K, Kim T, Tajiri T, Onda M, Sato S (1988) Modification of DNA by mitomycin C in cancer patients detected by ^{32}P-postlabeling analysis. Mutat Res 202:85
24. Kohn KW, Spears CL, Doty P (1966) Inter-strand crosslinking of DNA by nitrogen mustard. J Mol Biol 19:266
25. Kohn KW, Ewig RAG, Erickson LC, Zwelling LA (1980) Measurement of strand breaks and cross-links by alkaline elution. In: Rriedberg EC, Hanawalt PC (eds) DNA repair. Marcel Dekker, New York, p 379
26. Lepre CA, Lippard SJ (1990). Interaction of platinum antitumor compounds with DNA. Nucl. Acids Res Mol Biol 4:9
27. Li D, Xu D, Chandar N, Lombardi B, Randerath K (1990) Persistent reduction of indigenous DNA modification (I-compound) levels in liver DNA from male fisher rats fed choline-devoid diet and in DNA of resulting neoplasms. Cancer Res 50:7577
28. Lippard SJ (1982) New chemistry of an old molecule: cis-$(Pt(NH_3)_2Cl_2)$. Science 218:1075
29. Mehta JR (1980) Alkylation of guanosine and deoxyguanosine by phosphoramide mustard. Cancer Res 40:4183
30. Millard JT, Raucher S, Hopkins PB (1990) Mechlorethamine cross-links deoxyguanosine residual of 5′ GNC sequences in duplex DNA fragments. J Am Chem Soc 112:2459
31. Muller R, Rajewsky MF (1981) Immunological quantification by high-affinity antibodies of O^6-ethyldeoxyguanosine in DNA exposed to N-ethyl-N-nitrosourea. Cancer Res 40:887
32. Murray V, Motyka H, England PR, Wickham G, Lee HH, Denny WA, McFadyen WD (1992) An investigation of the sequence-specific interaction of cisplatin and four analogs, including two acridine-tethered complexes, with DNA inside human cells. Biochemistry 31:11812
33. Mustonen R, Hemminki K (1990) Application of the ^{32}P-postlabeling technique to detect DNA-adducts by cisplatin and methylating agents. Prog Clin Biol Res 340C:293
34. Mustonen R, Forsti R, Hietnen P, Hemminki K (1991) Measurement by ^{32}P-postlabeling of 7-methylguanine levels in white blood cell DNA of healthy individuals and cancer patients treated with dacarbazine and procarbazine. Human data and method development for 7-alkylguanines. Carcinogenesis 12:1423
35. Ojwang JD, Gruenberg DA, Loechler EL (1989) Synthesis of a duplex oligonucleotide containing a nitrogen mustard inter-strand DNA-DNA cross-link. Cancer Res 49:6529
36. Parker RJ, Gill I, Tarone R, Vionnet JA, Grunberg S, Muggis FM, Reed E (1991) Platinum-DNA damage in leukocytes of patients receiving carboplatin and cisplatin chemotherapy measured by atomic absorption spectroscopy. Carcinogenesis 12:1253
37. Randerath K, Reddy MV, Gupta RC (1981) ^{32}P-labeling test for DNA damage. Proc Natl Acad Sci USA 78:6126
38. Randerath K, Reddy R, Danna TF, Watson WP, Crane AE, Randerath E (1992) Formation of ribonucleotides in DNA modified by oxidative damage in vitro and in vivo, characterization by ^{32}P-postlabeling. Mutat Res 275:355
39. Reddy MV, Randerath K (1986) Nuclease P_1-mediated enhancement of sensitivity of ^{32}P-labeling test for structurally diverse DNA adducts. Carcinogenesis 7:1543
40. Reed E, Yuspa SH, Zwelling LA, Ozols RF, Poirier MC (1986) Quantitation of *cis*-diamminedichloroplatinum(II) (cis-platin)-DNA-intrastrand adducts in testicular and ovarian cancer patients receiving cisplatin chemotherapy. J Clin Invest 77:545
41. Routledge MN, Garner RC, Kirsch-Volders M (1992) Comparison ^{32}P-postlabeling and cytogenetic analysis of human blood treated in vitro with melphalan. Mutagenesis 7:329
42. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, p E3
43. Speit G, Menz W, Roscheisen C, Koberle B (1992) Cytogenetic and molecular characterization of the mutagenicity of chlorambucil in V79 cells. Mutat Res 283:75
44. Teicher BA (1992) Preclinical models for high dose therapy. In: Armitage JO, Antman KH (eds) High-dose cancer therapy: pharmacology, hematopoietins, stem cells. Williams & Wilkins, Baltimore, p 14
45. Terheggen PMAB, Floot BGJ, Scherer E, Beff AC, Fichtinger-Schepman AMJ, Englese LD (1987) Immunocytochemical detection of interaction products of cis-diamminedichloroplatinum (II) and cisdiammine-(1,1-cyclobutanedicarboxylato) platinum (II) with DNA in rodent tissue sections. Cancer Res 47:6719
46. Tilby MJ, Johnson C, Knox RJ, Cordell J, Roberts JJ, Dean C (1991) Sensitive detection of DNA modifications induced by cisplatin and carboplatin in vitro and in vivo using a monoclonal antibody. Cancer Res 51:123
47. Tomasz M (1970) Novel assay of 7-alkylation of guanine residues in DNA application to nitrogen mustard, triethylenemelamine and mitomycin C. Biochem Biophys 213:288
48. Wang Y, Herman TS, Teicher BA (1989) Platinum-dye complexes inhibit repair of potentially lethal damage following bleomycin treatment. Br J Cancer 59:722
49. Warpehoski MA, Harper DE, Mitchell MA, Monroe TJ (1992) Reversibility of the covalent reaction of cc-1065 and analogues with DNA. Biochemistry 31:2502
50. Weiland KL, Dooley TP (1991) In vitro and in vivo DNA bonding by the CC-1065 analogue U-73975. Biochemistry 30:7559
51. Yu D, Niu TQ, Austin-Ritchie P, Ludlum DB (1994) A ^{32}-P-postlabeling method for detecting unstable N-7-substituted deoxyguanosine adducts in DNA. Proc Nat Acad Sci USA 91:7232